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UvsW Protein Regulates Bacteriophage T4 Origin-Dependent Replication by Unwinding R-Loops

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The UvsW protein of bacteriophage T4 is involved in many aspects of phage DNA metabolism, including repair, recombination, and recombination-dependent replication. UvsW has also been implicated in the repression of origin-dependent replication at late times of infection, when UvsW is normally synthesized. Two well-characterized T4 origins, *ori*(*uvsY*) and *ori*(*34*), are believed to initiate replication through an R-loop mechanism. Here we provide both in vivo and in vitro evidence that UvsW is an RNA-DNA helicase that catalyzes the dissociation of RNA from origin R-loops. Two-dimensional gel analyses show that the replicative intermediates formed at *ori*(*uvsY*) persist longer in a *uvsW* mutant infection than in a wild-type infection. In addition, the inappropriate early expression of UvsW protein results in the loss of these replicative intermediates. Using a synthetic origin R-loop, we also demonstrate that purified UvsW functions as a helicase that efficiently dissociates RNA from R-loops. These and previous results from a number of studies provide strong evidence that UvsW is a molecular switch that allows T4 replication to progress from a mode that initiates from R-loops at origins to a mode that initiates from D-loops formed by recombination proteins.

Initiation of DNA replication from bacterial and eukaryotic origins generally involves limited unwinding within an A-T-rich region, promoted by either initiator protein(s) or an origin transcript (for reviews, see references 6, 8, and 16). The unwound region provides an assembly site for the replication complex including the replicative helicase, which catalyzes extensive unwinding of the parental strands. In plasmids containing the ColE1 replicon and in mitochondrial DNA (for reviews, see references 22 and 34), RNA polymerase (RNAP) generates an origin transcript that forms a persistent RNA-DNA hybrid (R-loop). The transcript holds open the origin region for assembly of the replisome and also serves as the primer for leading-strand synthesis.

Regulation of DNA replication is usually exerted at the stage of initiation. In the case of plasmid ColE1, regulation is achieved by expression of an anti-sense RNA (RNA I) that is complementary to the 5′ end of the primer RNA (RNA II). RNA I binds to RNA II and prevents it from folding into a conformation favorable for hybridization with the template (for reviews, see references 16 and 22). The detailed mechanism of regulation in both eukaryotic and prokaryotic chromosomal origins is not known, in part due to the complexities of the initiation events and of cell cycle regulation. In eukaryotic systems, a complex network of protein phosphorylation and dephosphorylation is involved in regulation. Although several eukaryotic replication proteins are phosphorylated (i.e., MCM proteins, DNA polymerase alpha-primase), the specific phosphorylation events that regulate initiation remain to be determined (for a review, see reference 37). In both eukaryotic and prokaryotic systems, proteins that bind and hydrolyze ATP are involved in the early steps of DNA replication (Cdc6p and DnaC, respectively), suggesting that ATP binding and hydrolysis may act as a molecular switch (for a review, see reference 25). This step may provide additional control by preventing reinitiation of replication at the origins, thus limiting replication to once per cell cycle.

Bacteriophage T4 uses two major replication initiation mechanisms, which are regulated to occur only during certain phases of the infective cycle (for reviews, see references 21 and 31). At early times of infection, replication proceeds mainly by the origin-dependent mode from any of several replication origins. Expression of phage-encoded recombination proteins results in the onset of recombination-dependent replication, which is the predominant mode during late times of infection. Throughout the phage life cycle, DNA replication is tightly coupled to transcription, recombination, and repair.

Modification of the *Escherichia coli* RNAP results in the temporal regulation of T4 gene expression (for a review, see reference 30). Immediately after infection, unmodified host RNAP recognizes T4 early promoters and begins the transcription of early genes. The early promoters are turned off and middle-mode promoters are activated upon expression of two phage-encoded proteins, AsiA (binds σ^{70}) and MotA (binds middle-mode promoters). Finally, upon synthesis of gp33 and gp55, the latter being a replacement σ factor (38), transcription is shifted from middle to late promoters.

Two well-characterized T4 origins of replication, *ori*(*uvsY*) and *ori*(*34*), consist of a middle-mode promoter juxtaposed to a DNA unwinding element (29). These origins have been cloned into a plasmid vector, and both replicate autonomously during T4 infection (17; for a review, see reference 21). Previous in vivo analyses using permanganate sensitivity provided a strong argument that the origin transcript forms an R-loop within the DNA unwinding element of plasmid-borne *ori*(*uvsY*) (4). Two-dimensional gel analyses of T4 genomic replication intermediates indicated that the transcript within the R-loop serves as the primer for leading-strand synthesis (1). Consistent with this

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model, putative RNA-DNA copolymers have been identified within the downstream region of *ori*(*uvsY*) (31, 35). Furthermore, a synthetic origin R-loop was recently shown to be an excellent substrate for the T4 in vitro replication system, with the RNA serving as the primer for leading-strand synthesis (31a).

Two models have been proposed for the repression of T4 origin-dependent replication at late times of infection. In the first model, origin-dependent replication is blocked when the host RNAP is converted to the late form, which cannot synthesize the origin transcript (28). The second model proposes that a phage-encoded protein produced late during T4 infection, UvsW, is a repressor of origin function that dissociates the origin R-loop (3, 7).

The earliest evidence that UvsW protein represses origin-dependent replication was that *uvsW* mutations (including nulls) restored the arrested DNA synthesis caused by mutations that inactivate T4 recombination proteins (5, 40, 41). Experiments with plasmid model systems suggested that the *uvsW* mutations did not restore the arrested DNA synthesis by directly rescuing recombination-dependent replication but rather by activating an alternative mode of late replication (7). The presence of five helicase motifs (including the Walker A motif) in the amino acid sequence of UvsW provided a strong hint that the protein functions as a helicase. Indeed, UvsW was purified as a glutathione *S*-transferase (GST) fusion protein and shown to exhibit both DNA-dependent ATPase and DNA helicase activities on branched substrates (3). Furthermore, a point mutation within the Walker A motif (K141R) eliminated both ATPase and helicase activities.

In this study, we demonstrate that one of the functions of the UvsW protein is to dissociate the origin R-loop through an RNA-DNA helicase activity. The dissolution of the R-loop leads to the switch from origin-dependent replication to recombination-dependent replication. Two-dimensional gel analyses show that the replicative intermediates formed at *ori*(*uvsY*) persist for a longer time during a *uvsW* mutant infection than in a wild-type T4 infection. The same method also shows that origin replicative intermediates are abolished when UvsW is produced artificially at early times. Finally, using a synthetic R-loop substrate, we show that purified UvsW efficiently dissociates the RNA from the R-loop.

MATERIALS AND METHODS

Materials. Restriction enzymes, *Taq* DNA polymerase, and T4 DNA ligase were purchased from various commercial sources, radiolabeled nucleotides were from Dupont NEN, and Nytran membranes were from Schleicher and Schuell. Oligonucleotides were synthesized by Sigma-Genosys Biotechnologies, Inc. GST-UvsW fusion proteins were purified as described by Carles-Kinch et al. (3). Luria broth (L broth) contained Bacto tryptone (Difco Laboratories) (10 g/liter), yeast extract (Difco Laboratories) (5 g/liter), and NaCl (10 g/liter).

***E. coli* and phage strains.** *E. coli* strains included CR63 (K12, *supD* λ^r) (9), BL21 (F^- *ompT hsd*S_B [r_B^- m_B^-] *gal dcm lon*), BL21 (DE3) (as BL21 with λ DE3), and AB1 [*araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsdR rpsL*], as described by Kreuzer et al. (19). Bacteriophage T4 strains included K10 (*amB262* [gene 38] *amS29* [gene 51] *nd28* [*denA*] *rIIPT8* [*denB-rII* deletion]) (33), K10-116 (as K10, with an 8-bp linker insertion within *uvsY* that renders the phage *uvsY*$^-$) (19), and K10-*uvsWΔ1* (as K10, with *uvsW*Δ*1*, a 1.3-kb *Hin*dIII deletion within *uvsW* that renders the phage *uvsW*$^-$) (7).

K10-*uvsW-K141R* is a derivative of K10 that harbors the *uvsW-K141R* point mutation (which also creates a *Bgl*II restriction site within the *uvsW* gene) (3). This phage was isolated using marker rescue from plasmid pKCK40. Phage containing mutations were identified by *Bgl*II digestion of *uvsW* PCR products (using primers UvsW-1 [5′-CGAAAATAGCTTTACCATGCG-3′] and UvsW-2 [5′-CGATTGTGAAGAGAGCACGTTCC-3′]).

Phage strain K10-*116 uvsW-K141R* [*ori*(*uvsY*)$^+$ *uvsY*$^-$ *uvsW-K141R*] was constructed by crossing phage K10-116 with K10-*uvsW-K141R* and screening individual plaques for the *uvsW* point mutation as described above. Plaques positive for the *uvsW* mutation were then screened for the *uvsY* mutation using *Eco*RV cleavage of a *uvsY* PCR product (using primers UvsY-1 [5′-CCCAATACTGC AACGAG-3′] and UvsY-2 [5′-AAGTCCATCCAATAACAACCAATAACAA CAATTG-3′]); the *uvsY* linker insertion creates a novel *Eco*RV site (19). K10-116-*uvsWΔ1* phage was constructed by a genetic cross between K10-116 and K10-*uvsWΔ1*, but in this case the PCR primers used to screen for the *uvsW* mutation were UvsW-A (5′-CACCGGGCACGGTATTAGG-3′) and UvsW-B (5′-TTCGTATCCTGGCCGACCA-3′).

Plasmids. Plasmid pKK405 is a pBR322 derivative harboring a 1.4-kb *Hin*dIII T4 fragment with *ori*(*uvsY*) (17, 18). pKCK41 is a pET11d derivative harboring the T4 *uvsW* gene under the control of a mutated T7 promoter (3). pKCK42 is a control plasmid for pKCK41 with a large fragment of the *uvsW* gene in the opposite orientation (relative to the promoter) (3). pKCK43 is a derivative of pKCK41 with the *uvsW* gene containing the K141R point mutation (3). pKCD444 is a derivative of pKCK41 with a *Cla*I deletion (*uvsW*Δ2) that removes most of the *uvsW* gene downstream of the promoter.

Two-dimensional agarose gel analysis. For the experiment shown in Fig. 2, BL21 cells were grown in L broth at 37°C to an optical density at 560 nm (OD_{560}) of 0.5 and then infected with K10 or K10-*uvsW-K141R* at a multiplicity of 6 PFU/cell. At the indicated time points, 1.5-ml aliquots were removed, cell pellets were collected by centrifugation, and total nucleic acids were purified as previously described (19). For the experiments shown in Fig. 4 and 5, BL21 (DE3) cells harboring the indicated plasmid were grown to an OD_{560} of 0.250 in L broth at 37°C, isopropyl-β-D-thiogalactopyranoside (IPTG) was added (100 μM), and the cells were further incubated at 37°C until the OD_{560} reached 0.5. T4 strain K10 was then added at a multiplicity of infection of 6 PFU/cell and samples were collected as described above.

DNA samples were digested with *Pac*I for 12 to 14 h at 37°C, followed by an additional 12 to 14 h of digestion at 25°C with *Swa*I. The conditions for two-dimensional gel electrophoresis (1, 2) were as follows. The first-dimension gel contained 0.4% agarose and was run in 0.5× TBE buffer (1× TBE buffer contained 89 mM Tris base, 89 mM boric acid, 2 mM Na_3EDTA) at 1 V/cm for 29 h. The second-dimension gel contained 1.0% agarose and ethidium bromide at 0.3 μg/ml; electrophoresis was at 4°C in 0.5× TBE buffer containing ethidium bromide (0.3 μg/ml) at 6 V/cm for 12 h (with buffer recirculation). Gels were analyzed by Southern blot hybridization, with a radioactive PCR fragment probe corresponding to T4 map coordinates 115,004 to 115,358 bp (T4 genome database, October 1998 release; probe prepared with the Random Primed kit [Roche Molecular Biochemicals]).

Northern blot analysis. RNA isolation and Northern blot analyses were performed as described by Belanger and Kreuzer (1). The oligonucleotide probe for the *uvsW* transcript, 5′-CGAAAATAGCTTTACCATGCG-3′, was 5′-end labeled using T4 kinase in the presence of [γ-^{32}P]dATP. After analysis of the *uvsW* transcript, the probe was washed from the blot, and the blot was reprobed with a ^{32}P-labeled oligonucleotide probe for the *ori*(*uvsY*) transcript, 5′-CCCAATA CTGCAACGAG-3′.

Transcription of RNA for the synthetic R-loop. The template for in vitro transcription reactions consisted of a PCR product with a mutated T7 promoter juxtaposed to *ori*(*uvsY*) DNA [positions −5 to +99 with respect to the *ori*(*uvsY*) promoter transcription] (29). The mutations in the T7 promoter were necessary to maintain complete sequence homology between the transcript and *ori*(*uvsY*) DNA. The PCRs included linear pKK405 DNA as the template in conjunction with specific primers T7−5 (5′-GCTTCGAAATTAATACGACTCACTATAG AATCTAAGTCCATCCAT-3′) and T7+99 (5′-GCGGTCGCGAATCTATAA ACACATCTTTCTTC-3′; *Bst*UI restriction site underlined). The two-stage PCR protocol consisted of the following steps: 94°C, 2 min; 45°C, 1 min; and 50°C, 3 min for 10 cycles in the first stage; and 94°C, 1 min; 60°C, 1 min; and 60°C, 2 min for 30 cycles in the second stage. The PCR products were gel purified and digested with *Bst*UI to generate a blunt-ended template for in vitro transcription reactions (Megashortscript kit; Ambion Company). The 104-base transcript was labeled by incorporation of [α-^{32}P]UTP residues, and the RNA concentration was determined by specific activity based on the nucleotide concentrations in the transcription reaction.

Preformed R-loop at *ori*(*uvsY*). The *ori*(*uvsY*) synthetic R-loop was generated by a modification of the protocol of Lee and Clayton (26). Briefly, 10 pmol of plasmid DNA was mixed with the indicated amount of *ori*(*uvsY*)-specific transcript in 100-μl reaction mixtures in R-loop buffer (62% formamide, 400 mM NaCl, 25 mM HEPES [pH 7.5], 1.25 mM EDTA). The mixture was incubated at

62°C for 4 h; the temperature was slowly reduced (1°C every 8.5 min) to 42°C, held at 42°C for 12 to 16 h, slowly cooled to 37°C (1°C per h), and held at 37°C for 1 h; and the mixture finally was incubated at room temperature for an additional 1 h. Where indicated, free RNA was separated from R-loop on a CL-4B Sepharose column equilibrated with R-loop buffer. R-loop-containing fractions were determined by radioactivity and by agarose gel electrophoresis followed by autoradiography, and fractions were stored at 4°C.

***Bgl*II inhibition assay.** The R-loop was monitored by inhibition of cleavage at a *Bgl*II site located 60 bp downstream of the *ori*(*uvsY*) transcription start site. Approximately 0.25 pmol of plasmid or R-loop was incubated at room temperature with 25 U of *Bgl*II for 1 h. The reaction was stopped with sodium dodecyl sulfate, and the products were applied to a 0.6% agarose gel (in 0.5× TBE buffer). Following electrophoresis, the DNA was analyzed by Southern blotting with a radioactive probe generated from pKK405 plasmid DNA using the Random Primed kit (Roche Molecular Biochemicals). Bands were quantitated using an AMBIS 100 Imager (AMBIS Inc.).

Mapping the RNA within the R-loop. The R-loop transcript was mapped within pKK405 by digestion with *Hin*dIII, which cuts the plasmid into two fragments. The R-loop (0.12 pmol) was first treated with 1 M glyoxal at 14°C for 2 h (14, 24) to modify the displaced strand, precipitated at −80°C for 2 h, and then incubated with *Hin*dIII (100 U) for 35 min at 37°C. The digests were analyzed by agarose gel electrophoresis followed by autoradiography.

R-loop unwinding assay. Formamide was removed from the R-loop by chromatography through a 1-ml Sephadex G-50 spin column. The unwinding reaction mixtures (20 μl) contained approximately 80 fmol of R-loop substrate in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM ATP (or as indicated in the Fig. 7 legend), bovine serum albumin at 100 μg/ml, and the indicated amounts of either purified GST-UvsW or GST–UvsW-K141R protein. After incubation for 15 min at 37°C, reactions were terminated by the addition of a solution containing 0.2% sodium dodecyl sulfate, 17 mM EDTA, 13% glycerol, and proteinase K (200 μg/ml). Reaction products were separated by electrophoresis through a 0.8% agarose gel with 1× TBE as the running buffer. The agarose gels were dried, and the products were visualized by autoradiography.

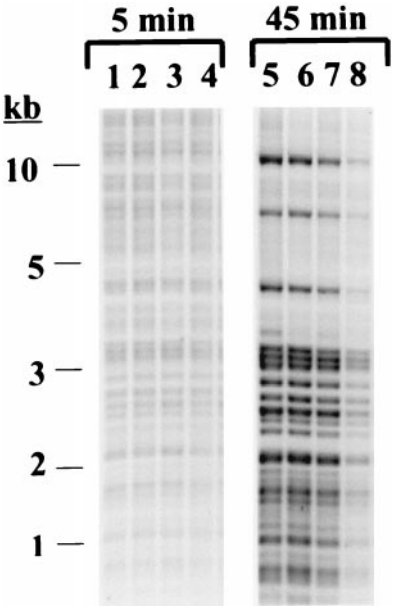

FIG. 1. DNA arrest phenotype is suppressed in a K10-*uvsW-K141R* phage infection. Total DNA was isolated from *E. coli* BL21 cells infected with K10 (lanes 1 and 5), K10-*116 uvsW-K141R* (lanes 2 and 6), K10-*116 uvsWΔ1* (lanes 3 and 7), or K10-*116* (*uvsY*$^-$, lanes 4 and 8) for 5 min (lanes 1 through 4) or 45 min (lanes 5 through 8); digested with *Ssp*I; and analyzed on a 0.8% agarose gel, which was stained with ethidium bromide. The size markers were generated from *Xba*I-digested T4dC DNA.

RESULTS

Characterization of *uvsW-K141R* phage. An amino acid substitution at position 141 (K→R) renders UvsW null for DNA-dependent ATPase and DNA helicase activities (3). Previous in vivo characterization of UvsW-K141R had only been performed using a plasmid that overexpresses the protein. We therefore began by substituting the *uvsW-K141R* mutation into the correct location in the phage genome by marker rescue from a plasmid, which involves homologous recombination, and then testing the well-characterized *uvsW* mutant phenotypes.

T4 phage with a mutation within a gene essential for recombination-dependent replication (i.e., genes 59, 46, and 47, *uvsX*, and *uvsY*) display a DNA arrest phenotype which can be suppressed by an additional mutation within *uvsW* (5, 40, 41). We tested the *uvsW-K141R* mutation for suppression of the DNA arrest phenotype by introducing it into a *uvsY*$^-$ background (K10-116) by a genetic cross. *E. coli* cells were infected with phage, total nucleic acids were isolated at 5 and 45 min postinfection, and the samples were digested with *Ssp*I. As expected, the *uvsY*$^-$ single mutant (K10-116) exhibited the DNA arrest phenotype, with very little replication between 5 and 45 min (Fig. 1, lanes 4 and 8). The DNA arrest phenotype was indeed suppressed by the *uvsW-K141R* mutation (Fig. 1, compare lanes 6 and 8), with the extent of suppression roughly equal to that of a complete knockout mutation (*uvsWΔ1*) (Fig. 1, compare lanes 6 and 7).

Phage containing mutations in *uvsW* also display increased sensitivity to hydroxyurea and 4′-(9-acridinylamino)methane sulfon-*m*-anisidide, with the latter being attributed to a defect in recombinational repair (12, 39). The *uvsW-K141R* phage exhibited the same hypersensitivity to both drugs as the *uvsW* deletion phage (data not shown). Therefore, within the context of the phage genome and with normal expression from the native late promoter, the K141R mutation causes the same phenotypes as a *uvsW* deletion phage.

Replication intermediates formed at *ori*(*uvsY*) persist during a *uvsW-K141R* infection. Two-dimensional gel analysis of the T4 *ori*(*uvsY*) region previously revealed a novel "comet" on the Y-arc (1). The comet consists of an accumulation of simple Y molecules with branch points in the region of the *ori*(*uvsY*) transcript. Formation of the comet was dependent on both the presence of the origin of replication and DNA synthesis. Various results implied that the Y molecules in the comet consist of intermediates in which unidirectional replication has been primed from the RNA of the origin R-loop. The fork thus formed has progressed out of the restriction fragment, but the second fork for bidirectional replication has not yet begun (1).

For the purpose of this next experiment, an important advantage of the *uvsW-K141R* phage is that the restriction enzyme cleavage pattern of the *ori*(*uvsY*) region is unaltered from the wild-type phage (not true for the *uvsW* deletion phage). Replicative intermediates were compared from a wild-type (K10) and a K10-*uvsW-K141R* mutant infection by two-dimensional gel analysis followed by Southern blotting for the *ori*(*uvsY*) fragment. The *uvsW*$^+$ infection demonstrated the characteristic comet structure (Fig. 2A, indicated by arrows), which was heavy at 9 min, faded by 11 min, and was absent thereafter. In the *uvsW-K141R* infection the replicative intermediates were prominent through 15 min postinfection (Fig. 2B), indicating that the replicative intermediates persist for a longer

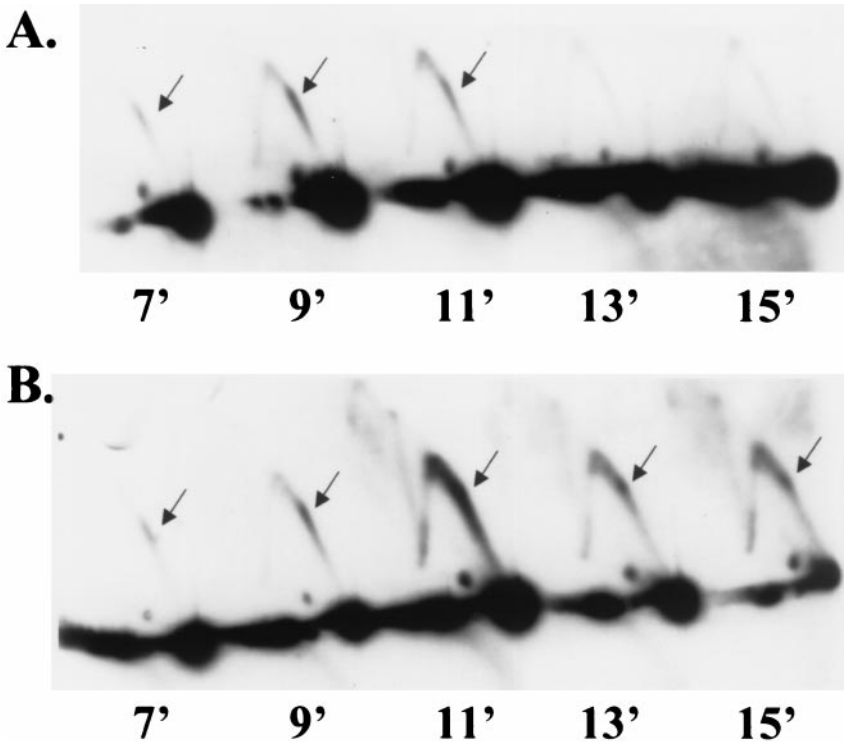

FIG. 2. The replicative intermediates generated at *ori*(*uvsY*) persist for a longer time during a K10-*uvsW-K141R* infection. Total genomic DNA was isolated from infections with phage K10 (A) and K10-*uvsW-K141R* (B) at the indicated times (minutes postinfection). All DNA samples were digested with *Pac*I and *Swa*I prior to electrophoresis. The first dimension of the two-dimension gel is represented horizontally (left to right) and the second dimension vertically (top to bottom). The gel was subjected to Southern blotting and probed with a DNA fragment that lies within *ori*(*uvsY*). Arrows indicate the comet on the simple Y-arc.

time in the *uvsW-K141R* infections. We also compared two-dimensional gel patterns of *ori*(*uvsY*) containing *Psi*I fragments from wild-type, *uvsW-K141R*, and *uvsWΔ1* time courses (this enzyme creates nearly identical 2.2- to 2.3-kb fragments from all three phage). The comet of replicative intermediates again persisted for a longer time in both the *uvsW-K141R* and the *uvsWΔ1* infections (data not shown). The most straightforward conclusion is that the wild-type UvsW protein represses origin replication by unwinding R-loops at late times, when the protein is expressed.

Because *uvsW* mutant phage have fairly serious growth deficiencies, we were concerned that the time course of the infection might be delayed in the *uvsW* mutant phage infection, which would compromise the conclusion just stated. To address this issue, we analyzed *uvsW* mRNA levels over time using Northern blot analysis. In both wild-type and *uvsW-K141R* infections, several *uvsW* transcripts were detected, with the largest consistent with the full-length transcript (Fig. 3A, arrow). The temporal appearance of *uvsW* mRNA was very similar in both infections, with a peak at about 13 min (Fig. 3A), when the origin is repressed in the *uvsW*$^+$ infection (Fig. 2A). These results confirm that gene expression in the two infections followed a similar time course. Furthermore, *uvsW* mRNA begins to be produced at precisely the time when the comet begins to fade in the wild-type infection (11 min), supporting the role of *uvsW* as the repressor of origin replication.

We also analyzed the *ori*(*uvsY*) transcript in this time course (Fig. 3B). As with the *uvsW* transcript, the temporal pattern of the *ori*(*uvsY*) transcript was similar in the two infections. The first appearance and the peak of the *ori*(*uvsY*) transcript were both significantly earlier than those of the *uvsW* transcript (Fig. 3A and B), as expected, since the *ori*(*uvsY*) transcript is produced from a middle (rather than a late) promoter. The *ori*(*uvsY*) transcript was abundant at 7 and 9 min, when the comet was strong, but peaked at 11 min, when the comet was fading in the wild-type infection (compare Fig. 2 and 3). The levels of *ori*(*uvsY*) transcript and comet roughly parallel each other throughout the time course of the *uvsW-K141R* infection.

Early expression of UvsW represses replication initiation at *ori*(*uvsY*). A previous study demonstrated that inappropriate early expression of UvsW blocked replication of an *ori*(*uvsY*)-containing plasmid during T4 infection (3). If UvsW represses T4 chromosomal origin function by unwinding the origin R-loop, then the early expression of UvsW should also abolish comet formation.

E. coli BL21 (DE3) cells harboring a plasmid which expresses UvsW from a T7 promoter, pKCK41, were treated with IPTG prior to infection to induce expression of the T7 RNAP and thus the UvsW protein. Two controls were included: (i) IPTG was omitted to prevent expression of UvsW, and (ii) IPTG was added to cells harboring pKCD444, a comparable plasmid with the *uvsWΔ2* deletion. In each case, the cells were infected with T4 strain K10 (*uvsW*$^+$) and DNA samples were analyzed by two-dimensional gel electrophoresis as a function of time after infection. Both the simple Y-arc and the comet structure were present at the expected times in the uninduced

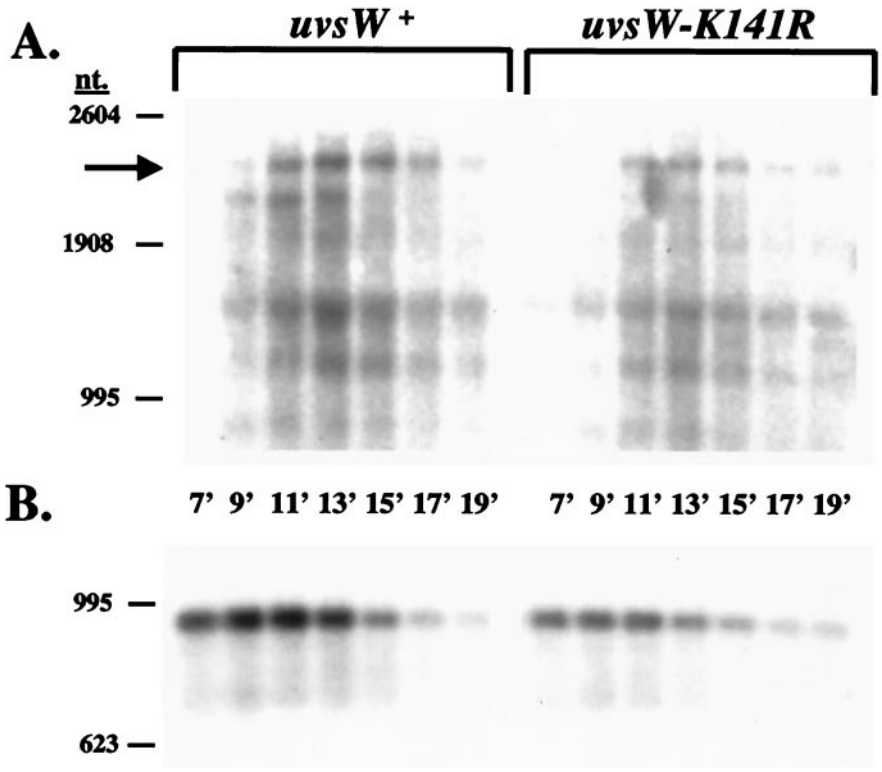

FIG. 3. *uvsW* and *uvsY* transcription are unaffected in the K10-*uvsW-K141R* infection. RNA was isolated from the same time courses as those for Fig. 2, separated by electrophoresis through a 1% denaturing (formaldehyde) agarose gel, and probed with specific oligonucleotides (see Materials and Methods) to detect the *uvsW* (A) and *ori*(*uvsY*) (B) transcripts. The infecting phage was K10 (left panels) or K10-*uvsW-K141R* (right panels). In panel A, the arrow represents the full-length *uvsW* transcript. In panel B, a faint but longer *uvsY* transcript was also detected (data not shown), consistent with the findings of Gruidl et al. (11). The RNA markers were from Promega, Inc. nt, nucleotides.

pKCK41 control samples (Fig. 4A) and the induced pKCD444 control (Fig. 4C). However, the comet and simple Y-arc were completely absent when UvsW had been expressed prior to phage infection (Fig. 4B). These results are consistent with the model that prematurely expressed UvsW unwinds the RNA transcript from the R-loop at *ori*(*uvsY*).

We also analyzed samples isolated from *E. coli* BL21 (DE3) cells containing plasmid pKCK43 (*uvsW-K141R*) or pKCK42 (most of *uvsW* in reverse orientation), induced for expression prior to the T4 K10 (*uvsW*$^+$) infection. As expected, the simple Y-arc and the comet were produced with the normal time course when the induced cells contained the plasmid with *uvsW* in the reverse orientation (Fig. 5B). The comet and simple Y-arc were also present when the UvsW-K141R protein was expressed prior to T4 infection (Fig. 5A). Interestingly, however, replicative intermediates from this infection were more intense and persisted longer than in any of the controls (compare Fig. 5A to Fig. 4A and B and 5B and C). In addition, the comet was more condensed, with a significant decrease in the length of the tail. A similar comet structure was previously detected when *E. coli* cells were infected with T4 under conditions devoid of RNase H activity, arguing that the 5′ end of the RNA is normally processed by RNase H (1). Therefore, the overexpressed UvsW-K141R protein apparently interferes with the processing of the 5′ end of the transcript by RNase H. A model to explain the prolonged existence of the comet will be presented in Discussion.

Generation of a synthetic R-loop at *ori*(*uvsY*). The above in vivo data strongly support our hypothesis that UvsW represses initiation of replication at *ori*(*uvsY*). Since UvsW has been shown to be a DNA helicase (3), we speculated that UvsW represses replication by unwinding the origin transcript from *ori*(*uvsY*). In order to test this model directly, we created a synthetic R-loop at *ori*(*uvsY*). The R-loop was formed by annealing an *ori*(*uvsY*)-specific transcript, generated with T7 RNAP, to a supercoiled plasmid pKK405, which harbors the T4 origin (see Materials and Methods). A stable R-loop was formed on supercoiled, but not linear, pKK405 plasmid (Fig. 6A, lanes 1 and 2, respectively), suggesting that negative superhelicity is necessary to stabilize the R-loop. As expected, R-loops were not formed on supercoiled pBR322, arguing that the RNA specifically hybridizes to *ori*(*uvsY*) in pKK405 (Fig. 6A, lane 3). Linearization of the R-loop plasmid by restriction enzyme digestion (at sites distal to the RNA) resulted in the loss of the RNA from the input plasmid DNA (data not shown), again consistent with negative superhelicity stabilizing the R-loop by favoring the unwound state of the two DNA strands.

Attempts to confirm that the RNA transcript was annealed to *ori*(*uvsY*) were hampered by the instability of the R-loop with restriction digestion. We therefore tested whether the R-loop could be stabilized by a mild treatment with glyoxal, which binds to G residues on single-stranded nucleic acids and should inhibit branch migration and thus displacement of the

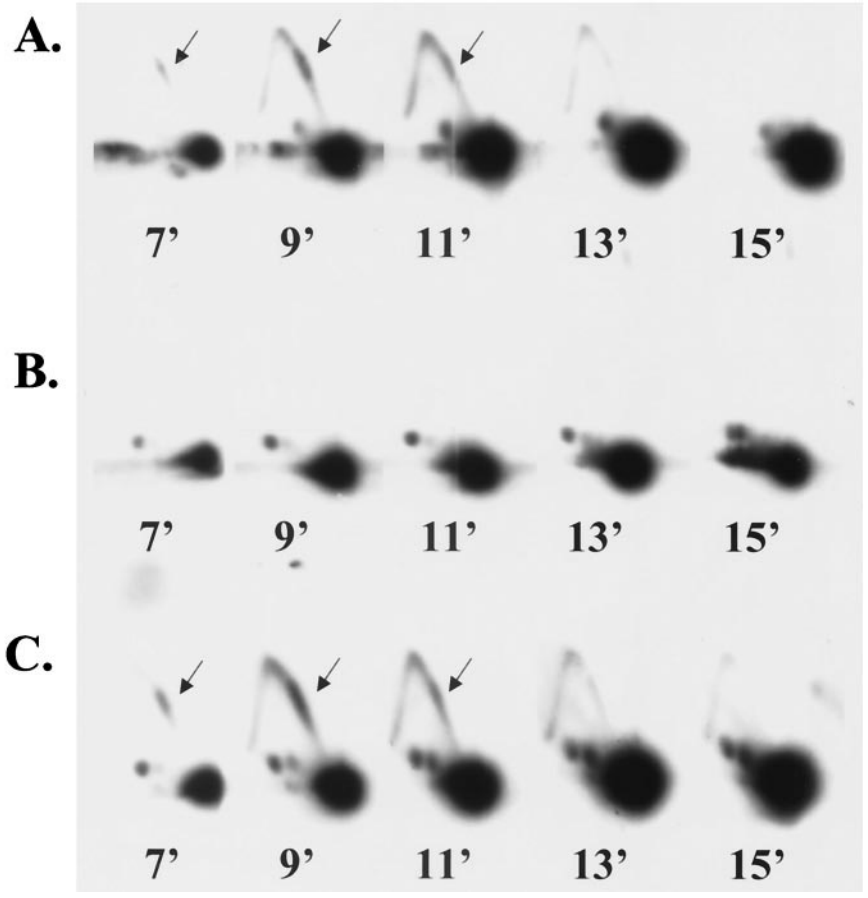

FIG. 4. *ori*(*uvsY*) is repressed by the inappropriate early expression of *uvsW*. *E. coli* cells harboring plasmid pKCK41 (*uvsW*$^+$) (A and B) or pKCD444 *(uvsWΔ2)* (C) were either induced with IPTG (B and C) or were not induced (A) prior to infection with phage strain K10. DNA samples were prepared at the indicated times postinfection and were analyzed by two-dimensional gel electrophoresis as described for Fig. 2. The arrows indicate the comet region on the simple Y-arc.

RNA (14, 24). The synthetic R-loop was treated with glyoxal under very mild reaction conditions to minimize denaturation of duplex DNA and then was treated with *Hin*dIII. As expected, two *Hin*dIII fragments were detected by ethidium bromide staining after gel electrophoresis, a 4.0-kb vector fragment and a 1.4-kb *ori*(*uvsY*)-containing fragment (data not shown). Only the 1.4-kb *Hin*dIII fragment was labeled with the radioactive RNA (Fig. 6B, lane 3), confirming that the transcript was annealed to *ori*(*uvsY*).

To establish an optimal RNA/DNA ratio for R-loop formation and to provide additional evidence for the correct R-loop, we took advantage of a unique *Bgl*II restriction site located 60 bp downstream of the transcription start site. *Bgl*II digestion is blocked when a stable R-loop is formed at its cleavage site, and therefore the percentage of target DNA containing stable R-loops can be quantified by the amount of supercoiled (Fig. 6C, sc, uncut by *Bgl*II) versus linear (Fig. 6C, l, *Bgl*II-cleaved) DNA. R-loops formed efficiently at the two highest RNA/DNA ratios, with approximately 80% of the input plasmid DNA converted to R-loop at a ratio of 100 pmol of RNA to 10 pmol of plasmid (Fig. 6C).

UvsW can unwind RNA from a synthetic R-loop. The synthetic R-loop provided a substrate to test the potential RNA-DNA helicase activity of UvsW. Purified GST-UvsW fusion protein was previously shown to be active in both ATPase and DNA helicase activities, while the mutant GST–UvsW-K141R was found to be inactive in both assays (3). We first measured R-loop dissociation with increasing concentrations of either GST-UvsW or GST–UvsW-K141R proteins in the presence or absence of ATP. Wild-type GST-UvsW dissociated the RNA from the R-loop in an ATP-dependent reaction (Fig. 7A, compare lanes 1 through 4 to lanes 5 through 8). As with the previous DNA helicase assays, the GST–UvsW-K141R protein was completely inactive (Fig. 7B). Because dissociation of RNA from the R-loop by GST-UvsW was dependent on ATP (Fig. 7A) and $MgCl_2$ (data not shown), the dissociation is apparently a true helicase activity and not just a helix destabilizing activity.

DISCUSSION

In this study we have analyzed the role of the T4 UvsW protein in the repression of origin-dependent replication at late times of infection. Based on our results, we conclude that UvsW represses T4 origins by removing the RNA from origin R-loops via an RNA-DNA helicase activity. First, the origin replicative intermediates disappear much faster in a wild-type infection (*uvsW*$^+$) than in a *uvsW* mutant infection. In the

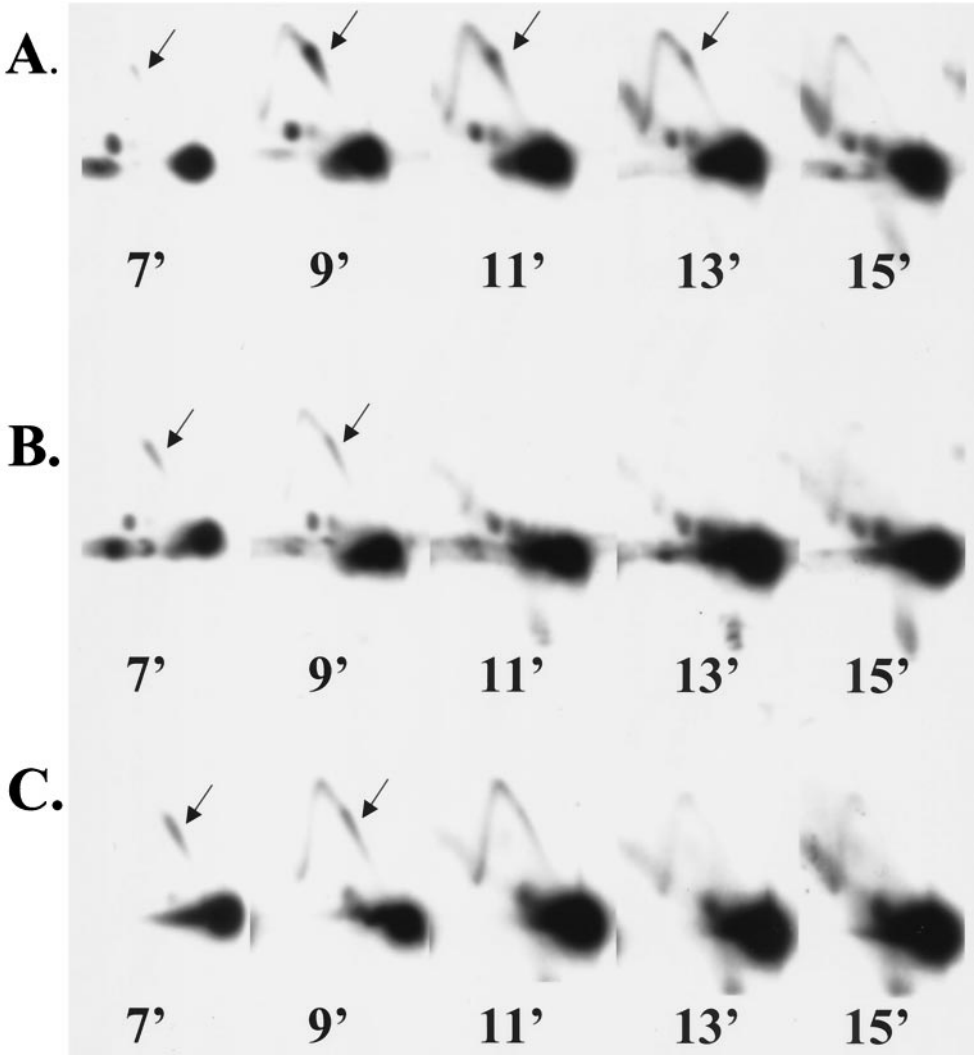

FIG. 5. The inappropriate early expression of *uvsW* mutant protein alters replication from *ori*(*uvsY*). *E. coli* cells harboring plasmid pKCK43 (*uvsW-K141R*) (A), pKCK42 (*uvsW* with large inversion) (B), or pKCD444 *(uvsWΔ2)* (C) were induced with IPTG prior to infection with phage K10. DNA samples were prepared at the indicated time points and analyzed by two-dimensional gel electrophoresis as described for Fig. 2. The arrows indicate the comet region on the simple Y-arc.

wild-type infection, the disappearance of the replicative intermediates correlated closely with the appearance of the *uvsW* mRNA. Second, the expression of wild-type UvsW protein prior to T4 infection prevented formation of replicative intermediates at the T4 origin. Third, purified GST-UvsW but not GST–UvsW-K141R protein effectively unwound the RNA from a synthetic origin R-loop. These results provide strong evidence to distinguish between two previous models: (i) origins are repressed at the transcriptional level by modification of the host RNAP into its late form (28), and (ii) UvsW actively represses origin function (3, 7).

Our two-dimensional gel analysis of wild-type and *uvsW* mutant infections, in conjunction with Northern blot analysis, strongly argues that the temporal regulation of gene expression does not play a significant role in repressing origin-dependent replication in the wild-type infection. The temporal appearance of the *uvsY* (middle) and *uvsW* (late) transcripts was identical between the $uvsW^+$ and $uvsW^-$ infections, indicating that the regulation of gene expression was the same in both infections. As mentioned above, the comet, consisting of accumulated replicative intermediates, disappeared just as the late *uvsW* message was increasing in the wild-type infection. Thereafter, the comet was absent for the remainder of the infection, even though origin transcripts were still abundant for some time. If not for the production of UvsW protein, these origin transcripts that are present at late times could be capable of initiating replication, as evidenced by the prolonged appearance of the comet in the *uvsW* mutant infection. Indeed, the temporal appearance of the comet in the *uvsW* mutant infection roughly paralleled that of the origin transcript. At present, we do not know if origin transcripts are still being actively synthesized at these late times, indicating a mixture of middle- and late-mode RNAP in the cell, or if middle-mode transcription is shut off but the origin transcripts have a long half-life.

We found a condensed comet that persisted for a longer time when UvsW-K141R was overexpressed prior to T4 infec-

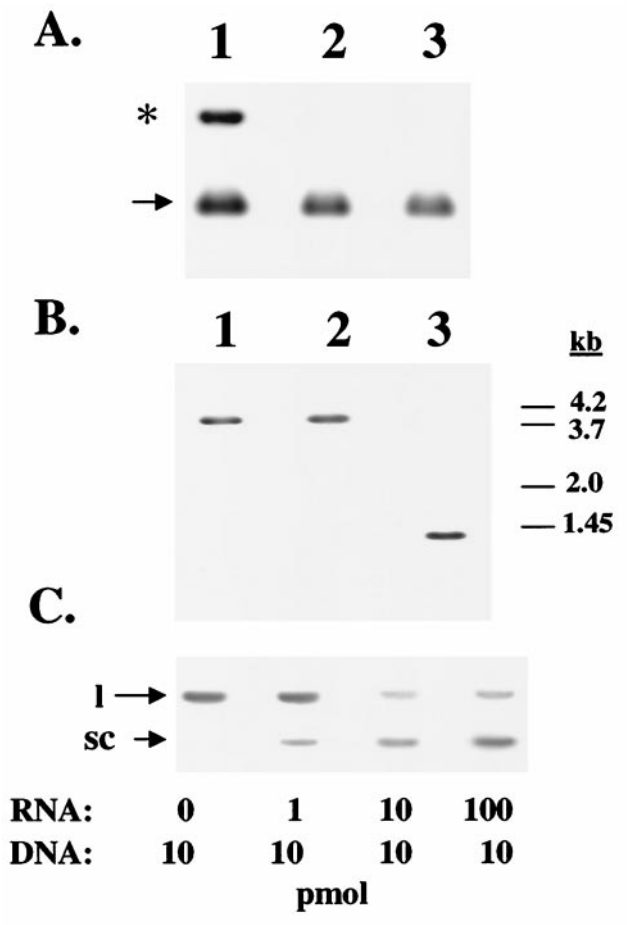

FIG. 6. A synthetic R-loop can be formed at *ori*(*uvsY*). (A) The formation of an R-loop was tested on supercoiled (lane 1) or linearized (lane 2) pKK405 [pBR322 harboring *ori*(*uvsY*)] or supercoiled pBR322 (lane 3) at a ratio of 100 pmol of ^{32}P-labeled RNA to 10 pmol of DNA. Products were separated by electrophoresis through a 0.8% agarose gel and visualized by autoradiography. The R-loop migrates at the position of free plasmid DNA in the agarose gel (data not shown). The positions of the free RNA (arrow) and R-loop (*) are indicated. (B) Mapping of RNA to *ori*(*uvsY*). R-loop was generated with ^{32}P-labeled RNA, purified away from free RNA, and treated with *Hin*dIII after fixation of the displaced strand with glyoxal (see Materials and Methods). The untreated R-loop is shown in lane 1, R-loop treated with glyoxal only is shown in lane 2, and R-loop treated with glyoxal and *Hin*dIII is shown in lane 3. *Hin*dIII cleaves pKK405 into a 4.4-kb vector fragment and a 1.38-kb *ori*(*uvsY*) insert, and only the latter contained the RNA label. The size markers were generated from *Xba*I-digested T4dC DNA. (C) R-loop formation was optimized by varying the ratio of RNA to DNA during the assembly protocol. The samples were treated with *Bgl*II, which cleaves free DNA but not R-loops (see Materials and Methods). The linear (l; cleaved) and supercoiled (sc; uncleaved) products were separated by electrophoresis through a 0.8% agarose gel and visualized by Southern blotting. Maximal R-loop formation was obtained at a ratio of 100 pmol of RNA to 10 pmol of DNA.

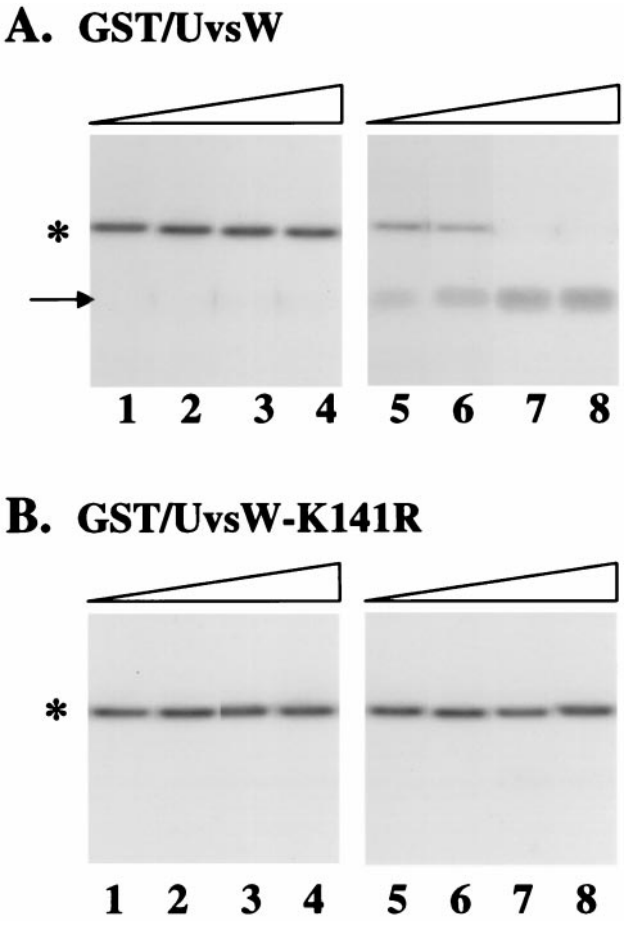

FIG. 7. UvsW displays an RNA-DNA helicase activity. Unwinding reaction mixtures contained 80 fmol of radioactive R-loop and the following increasing amounts of either GST-UvsW (A) or GST–UvsW-K141R (B): 57 fmol (lanes 1 and 5); 115 fmol (lanes 2 and 6); 217 fmol (lanes 3 and 7); and 576 fmol (lanes 4 and 8). ATP was absent in lanes 1 through 4 and was present in lanes 5 through 8. All products were separated by electrophoresis through a 0.8% agarose gel and visualized by autoradiography. The positions of the free RNA (arrow) and R-loop (*) are indicated.

tion. The comet in the wild-type infection consists of replicative intermediates in which unidirectional replication has been primed from the RNA of the origin R-loop (1). The fork thus formed has progressed out of the restriction fragment, but the leftward fork for bidirectional replication has not yet begun. The comet was also condensed into a spot in an RNase H-deficient infection, arguing that RNase H often processes the 5′ end of the origin transcript in the wild-type infection (1). The generation of the condensed comet with overexpressed UvsW-K141R therefore suggests that binding of UvsW-K141R to the R-loop prevents 5′-end processing by RNase H. A model to explain the persistence of the comet is that the overproduced UvsW-K141R protein also prevents R-loop unwinding by the wild-type protein made from the infecting phage genome.

The two-dimensional gel analysis is limited in defining the precise role of UvsW at the origin, since it does not assay R-loops directly but rather provides a snapshot of origin replication intermediates. The development of the synthetic R-loop at *ori*(*uvsY*) allows us to mimic the initial intermediate of replication during in vitro experiments. This R-loop has recently been used as a substrate for in vitro replication assays using purified T4 proteins (31a). The RNA transcript served as a very efficient primer for leading-strand synthesis, strongly validating previous in vivo data. The unwinding experiments described here demonstrate that UvsW can dissociate the

RNA from the R-loop substrate via RNA-DNA helicase activity, providing biochemical evidence that UvsW represses the origins by removing the RNA from the origin R-loop.

The *E. coli* RecG protein is a functional analog of UvsW. Both proteins are involved in recombination and repair and demonstrate helicase activity on branched DNA substrates (3, 27). Like UvsW, RecG has been shown to inhibit replication and unwind R-loops (10, 32, 36). Thus, overexpression of RecG reduces the copy number of plasmids harboring the ColE1 replicon, and the ability of RecG to unwind R-loops is correlated to its ability to reduce copy number. Futhermore, an *E. coli recG* mutant, like an *rnhA* mutant, displays constitutive stable DNA replication (cSDR, *oriC*-independent replication), which is thought to initiate from artificially stabilized R-loops (13, 15). Although *E. coli recG* or *rnhA* single mutants survive, the double mutant is nonviable, indicating that accumulation of excess R-loops somehow leads to cell death (13). The strongest evidence that UvsW is a functional analog of *E. coli* RecG is that the growth of the *recG rnhA* double mutant can be rescued by the expression of UvsW (3). With the results described here, we conclude that UvsW rescues the double mutant by alleviating the accumulation of R-loops. It is interesting to speculate that replication from R-loop substrates is a primordial replication system which has become repressed in most systems, perhaps because it is difficult to coordinate with cell division and growth.

Another interesting point is that RecG, which is present prior to T4 infection, does not block origin R-loop formation during T4 infection. Synthesis of new RecG protein is presumably shut off upon T4 infection through the arrest of host transcription and translation (for a review, see reference 23). Perhaps pre-existing RecG protein is also inactivated, for example, by covalent modification. Another possibility is that coating of the displaced single strand of the R-loop by the T4-encoded single-strand DNA binding protein, gp32, blocks RecG from binding (but does not interfere with binding and unwinding by UvsW).

One of the most important phenotypes of a *uvsW* mutant phage is that the arrested DNA synthesis normally caused by mutations that block recombination-dependent replication is restored (5, 40, 41). Previous experiments using plasmid model systems indicated that a *uvsW* mutation does not restore the arrested DNA synthesis by directly rescuing recombination-dependent replication but rather activates an alternative mode of late replication involving replication origins (7). Based on our analysis of *ori*(*uvsY*) here, this alternative mode of replication could simply depend on the persistence of R-loops at the known T4 origins even after middle-mode transcription is blocked (i.e., long-lived origin transcripts). In addition, the UvsW protein may normally remove RNA from R-loops at late promoters, which would thus qualify as cryptic origins that are only active in *uvsW* mutant infections.

In summary, the conclusion of this study is that UvsW, a protein expressed late during T4 infection, is an RNA-DNA helicase that represses origin-dependent replication by unwinding R-loops. In addition to repressing origin DNA replication, UvsW very likely promotes the process of recombination-dependent replication that becomes dominant at late times of infection (for a review, see reference 20). Thus, UvsW functions as a molecular switch, changing the substrate for T4 replication from R-loops to D-loops. Although the precise role of UvsW in recombination-dependent replication is not known, one reasonable model is that the protein promotes strand invasion to form D-loops.

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